# GD3/proteosome vaccines induce consistent IgM antibodies against the ganglioside GD3

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*The gangliosides of melanoma and other tumours of neuroectodermal origin are suitable targets for immune intervention with tumour vaccines. The optimal vaccines in current use contain ganglioside plus bacillus Calmette–Guérin and induce considerable morbidity. We have screened a variety of new adjuvants in the mouse, and describe one antigen-delivery system, proteosomes, which is especially effective. Highly hydrophobic Neisserial outer membrane proteins (OMP) form multimolecular liposome-like vesicular structures termed proteosomes which can readily incorporate amphiphilic molecules such as GD3 ganglioside. The optimal GD3/proteosome vaccine formulation for induction of GD3 antibodies in the mouse is determined. Interestingly, the use of potent immunological adjuvants in addition to proteosomes augments the IgM and IgG antibody titres against OMP in these vaccines but GD3 antibody titres are unaffected. The application of proteosomes to enhance the immune response to GD3 extends the concept of the proteosome immunopotentiating system from lipopeptides to amphipathic carbohydrate epitopes such as cell-surface gangliosides. The demonstrated safety of meningococcal OMP in humans and the data in mice presented here suggest that proteosome vaccines have potential for augmenting the immunogenicity of amphipathic tumour antigens in humans.*

**Keywords:** Proteosomes; antigen-delivery system; gangliosides; tumour

The gangliosides GM2, GD2, GD3, 9-*O*-acetyl GD3 and GT3 are expressed on the cell surface of human malignant melanoma and other tumours of neuroectodermal origin[1–4]. As cell-surface antigens, they are inserted in cell membranes via their ceramide moiety while the carbohydrate portion of the molecule remains outside the cell and exposed. The importance of these gangliosides as potential targets for active specific immunotherapy with tumour vaccines has been suggested by (1) their limited expression on most normal tissues[4,5], (2) regression of melanoma and neuroblastoma metastases in some patients treated with anti-GD3 and anti-GD2 murine monoclonal antibodies (MmAb)[6,7] and (3) a prolonged disease-free interval and survival in patients with GM2 antibodies after immunization with vaccines containing GM2[8]. We have previously compared a variety of approaches to augmenting the immunogenicity of gangliosides and described a killed *Salmonella minnesota* mutant preparation (R595) as the most effective adjuvant in the mouse[5,9]. The effectiveness of R595 as an adjuvant in humans is much lower than in the mouse[10], presumably because the active ingredient of R595 (endotoxin) is a B-cell mitogen in the mouse but not in humans. Bacillus Calmette–Guérin (BCG) plus GM2, on the other hand, has been an effective human vaccine, producing an IgM antibody response $\geq 1/40$ against GM2 in 87% of patients immunized and IgG antibodies in occasional patients[8]. However, a high titre ($\geq 1/160$) antibody response against GM2 was seen in only 40% of patients, no consistent response was seen against GD2 or GD3 after immunization with BCG plus these gangliosides in patients, and significant morbidity is associated with local inflammatory reactions resulting from BCG. Consequently, we have continued to screen new adjuvants, focusing especially on adjuvants which may be B-cell mitogens in humans.

We test here a variety of new adjuvants, selecting for further study one, proteosomes, which is as effective as R595 when administered subcutaneously in the mouse and which is a B-cell mitogen in both the mouse and humans. Proteosome is a term used to describe preparations of the highly hydrophobic outer membrane proteins (OMP) of *Neisseria meningitidis* or *Neisseria gonorrhoeae* that naturally form liposome-like multimolecular vesicular structures[11–13]. These readily incorporate antigens containing hydrophobic anchor moieties. Proteosomes were considered as potential adjuvants since OMP are potent T cell-dependent

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antigens and B-cell mitogens in both conventional and lipopolysaccharide (LPS) hyporesponsive C3H/HeJ mice and in humans[13,14]. They have been shown to initiate or augment IgG antibody responses against peptides when hydrophobically complexed to them via lipid moieties present at the peptide's amino or carboxyl terminus[11-13]. In addition, although meningococcal group B polysaccharide is not immunogenic alone, immunization with this antigen hydrophobically complexed to OMP induces anti-B polysaccharide antibodies in humans[14]. Moreover, vaccines containing OMP preparations similar to the proteosomes used in this study have been safely given to over 25 000 humans in clinical trials designed to test meningococcal vaccines[13,14]. Having identified here proteosomes as a particularly potent adjuvant for immunization against GD3 in the mouse, the use of various proteosome formulations in preparation for clinical studies and the nature of the antibody response induced, are explored.

## METHODS

### Reagents

Glycolipids GM3, GM2, GM1, GD1b and GD3 were provided by Fidia Research Laboratories (Abano Terme, Italy). GD2 was prepared by treatment of GD1b with bull testis $\beta$-galactosidase as previously described[15].

### Vaccines

Vaccines were prepared by mixing GD3 and adjuvant immediately before immunizations or as described below. The adjuvant dose used was that described by the authors or others as effective (see references below). Vaccines contained 30 $\mu$g of GD3 plus *S. minnesota* mutant R595 300 $\mu$g (see References 4 and 9 for preparation procedure), pokeweed mitogen (PWM) 0.1 ml[16] (Gibco Laboratories, Grand Island, NY), SAF-m containing threonyl MDP 50 $\mu$g and pleuronic block copolymer L121[17] (Syntex Corporation, Palo Alto, CA), QS21 containing a saponin Quil A fraction 10 $\mu$g[18] (Cambridge Bioscience, Worcester, MA), concanavalin A (con A) 10 $\mu$g[19] (Pharmacia, Uppsala, Sweden), Detox containing BCG cell-wall skeletons and monophosphoryl lipid A (MPLA) 125/12.5 $\mu$g[9] (Ribi Immunochem, Hamilton, MT), and small unilamellar liposomes prepared from lecithin plus GD3 (5/1 by weight) forced through a 0.1 $\mu$m sieve as previously described[9] and used alone or sonicated for 2 min in a Bronson 1200 water bath sonicator (Shelton, CT) with 50 $\mu$g MPLA or 15 $\mu$g OMP.

### Proteosomes

Preparation and purification of the highly hydrophobic and normally insoluble OMP were performed (1) from *N. meningitidis* (Nm) strain 8047 (B:2b:P1.2) at the Walter Reed Army Institute of Research (WR) by W.D.Z. as previously described[11,12]; (2) from Nm 8765 (B15:P1.3) or *N. gonorrhoeae* UU1X P1A at Pasteur Mérieux (PM) as previously described[20]; or (3) from Nm 8529 (B15:P1.3) by Connaught Laboratories (CL) (Swiftwater, PA). WR and CL preparations contained $\approx$70% porins while PM preparations contained 99% porins. WR proteosomes were used in all experiments unless otherwise indicated. Proteosome/ganglioside complexes are formed by mixing OMP ganglioside in 1% empigen and then dialysing across a 100 kDa cut-off membrane (Spectra-pore) for 10 days to remove all detergent as previously described[11]. During this process, ganglioside incorporates into the vesicular interface of the proteosomes via its hydrophobic ceramide moiety (it can be precipitated with the proteosomes by ultracentrifugation at 100 000$g$ for 1 h) allowing the carbohydrate component to form a hydrophilic external environment that effectively confers solubility to the proteosomes in phosphate-buffered saline (PBS). The ratio by weight of ganglioside to protein in each batch of proteosomes was confirmed by the method of Lowry *et al.*[21] and by sialic acid determinations[22], and was 2/1 unless otherwise indicated. Porin content of proteosomes was determined by Western blot with 2D1, a porin-specific murine monoclonal antibody (M.S. Blake, unpublished results). They were used as formed or passed through a 1.0 or 0.1 $\mu$m extruder filter (Lipex Biomembranes Inc., Vancouver, BC, Canada). Proteosomes were aliquoted, dried under nitrogen and stored at 4°C.

### Vaccine administration

In each experiment (generally 50–60 mice) five mice selected randomly from the same shipment were immunized with a given vaccine three times. The vaccines were administered subcutaneously 2 weeks apart in a total volume of 0.1 ml/mouse. One mouse died 4 days after receiving a vaccine containing SAF-m and all mice injected with SAF-m developed local drainage and scab formation lasting 1–2 weeks. No other toxicity or morbidity was detected as a consequence of vaccine administration. This vaccination schedule with cyclophosphamide (Cy) pretreatment 3 days before the first vaccination (15 mg kg$^{-1}$) is based on our previous studies with ganglioside vaccines[5,9] and was not varied in these experiments.

### Animals

Female Balb/c-C57BL/6F1 mice, 6 weeks of age, were obtained from the Jackson Laboratory, Bar Harbor, ME. Immunization was begun within 2–4 weeks.

### Serological assays

Mice were bled from the retro-orbital sinus before and 2 weeks after each vaccination and serum samples for serological testing (approximately 0.1 ml) were stored at −20°C. ELISAs were performed using rabbit anti-mouse IgM and IgG linked to alkaline phosphatase (Zymed, San Francisco, CA) as previously described[5,9]. The absorbance ($A$) with pretreatment mouse serum was subtracted from post-treatment values to yield the experimental values at each titre. To eliminate the effect of non-specific 'sticky' sera, sera were also tested on plates which were processed identically but to which no ganglioside had been added. The absorbance at each titre obtained on this plate was subtracted from the experimental value, yielding the corrected absorbance. Serological titre in ELISA is defined as the highest dilution yielding a corrected $A \geq 0.10$. All assays were repeated on two or more occasions to ensure consistency. Immune stains (dot blots) were performed as previously described[23] with slight modifications. In brief, 0.5 $\mu$g

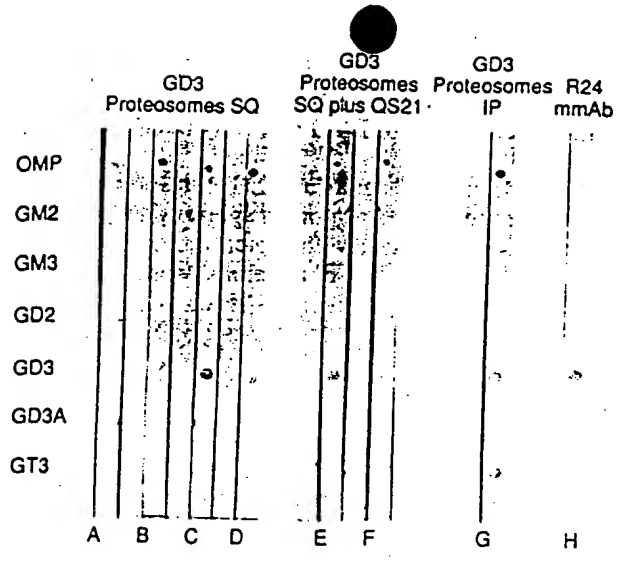


**Figure 1** Detection of GD3 antibody in pre- and postimmunization sera from seven mice immunized with GD3/proteosome vaccines, demonstrated by dot blot immune staining. Ganglioside standards were applied to nitrocellulose strips and allowed to react with sera from individual mice and peroxidase-labelled goat anti-mouse IgM antibody. Reactions are graded as indicated. No serum reacted with any antigen before immunization. The reactions were graded as follows: mouse A–OMP 2+, GD3 2+; mouse B–OMP 3+, GD3 2+; mouse C–OMP 2+, GD3 3+; mouse D–OMP 3+, GD3 2+; mouse E–OMP 3+, GD3 2+; mouse F–OMP 3+, GD3 2+; mouse G–OMP 3+, GD3 3+, GD3 amide 1+, GT3 3+; R24 murine monoclonal antibody positive control GD3 3+, GD3 amide 2+, GT3 3+. SQ, subcutaneous; IP, intraperitoneal

**Table 1** Serological response to GD3/adjuvant vaccines

| Vaccine[a] | No. of mice | Anti-GD3 ELISA titres (*n*) | Anti-GD dot blot immune stain results |
|---|---|---|---|
| GD3 | 10 | 0 | 0 |
| GD3/PWM | 5 | 0 | 0 |
| GD3/Con A | 5 | 0 | 0 |
| GD3/SAF-m | 5 | 0 | 0 |
| GD3/QS21 | 5 | 20(2), 0(3) | 0 |
| GD3/Detox | 5 | 40(3), 0(2) | 1+, 0(4) |
| GD3/liposomes | 5 | 40(2), 0(3) | 1+, 0(4) |
| GD3/liposomes/MPLA | 5 | 80, 40(3), 0 | 2+(2), 1+(2), 0 |
| GD3/liposomes/OMP[b] | 15 | 80, 40(4), 0(10) | 2+, 1+(2), 0(12) |
| GD3/OMP[b] | 10 | 40(3), 20, 0(6) | 1+(2), 0(8) |
| GD3/R595 | 10 | 1280(3), 640, 320(2), 160, 80(2), 40 | 3+(3), 2+(4), 1+, 0(2) |
| GD3/proteosomes (2/1) | 10 | 1280(2), 640, 320(2), 160, 80(2), 0(2) | 3+(4), 2+(4), 1+, 0 |

[a]Mice were immunized three times at 2-week intervals, subcutaneously. Vaccines contained 30 μg GD3
[b]OMP was solubilized by sonication with GD3 in saline as opposed to the standard, more complex procedure for proteosome formation described in Methods

ganglioside was spotted on nitrocellulose paper strips. The strips were blocked at room temperature for 2 h in PBS containing 3% bovine serum albumin (BSA). The serum was diluted 1/150 with 3% BSA in PBS and the strips were incubated with the diluted serum in Accutron trays (Schleicher and Schuell, Keene, NH) at room temperature for 16 h. Strips were washed five times with PBS mixture and incubated for 5 h with horseradish peroxidase-conjugated anti-mouse IgM antibody diluted 1:200 (Zymed, San Francisco, CA). Peroxidase staining was performed and quantified as negative, 1+, 2+ or 3+ as shown in *Figure 1*. Complement-dependent cytotoxicity assays with guinea-pig complement sera were performed on melanoma cell line SK MEL-28 as previously described[10].

## RESULTS

### Effects of various adjuvants on the immunogenicity of GD3: selection of proteosomes for further study

Vaccines were prepared with GD3 alone or GD3 plus various adjuvants using the preparation procedures described by the authors or others as optimal. The results are shown in *Table 1*. R595 and proteosomes resulted in consistent IgM antibody responses against GD3. IgG antibody responses were not seen with any preparation. Based on these results, attention was focused on GD3/proteosome vaccine preparations.

### Effect of proteosome formulation on GD3 immunogenicity

In separate experiments proteosomes were prepared in various ways, from various sources, with various GD3/proteosome ratios, and after passage through pores of various sizes. OMP solubilized with GD3 by sonication (as opposed to the standard method for proteosome formation) was poorly immunogenic (*Table 1*). Proteosomes containing GD3/OMP ratios between 2/1 and 1/1 were found to be most effective and passage through 0.1 or 1 μm pores resulted in no change in immunogenicity (see *Table 2*). GD3/proteosomes were also mixed with alum or with each of the adjuvants used in *Table 1* and administered subcutaneously. No augmentation of the antibody response against GD3 was seen (data not shown). Intraperitoneal immunization generally resulted in two–fourfold higher titres of IgM antibodies and occasionally IgG antibodies, but as this is not a clinically practical immunization route, this approach was not pursued. OMP prepared from Nm strains 8047 (B:2b:P1.2), and 8765 (B:15:P1.3), and

**Table 2** Effect of proteosome formulation on serological response

| Vaccine | No. of mice | Median GD3 ELISA titre | Median GD3 immune stain response |
|---|---|---|---|
| GD3/proteosome ratio | | | |
| 9/1 | 5 | 0 | 0 |
| 3/1 | 10 | 40 | 2+ |
| 2/1 | 10 | 320 | 3+ |
| 2/1 (i.p.) | 5 | 640 | 3+ |
| 1/1 | 10 | 80 | 2+ |
| 1/2 | 5 | 20 | 2+ |
| 1/3 | 5 | 0 | 1+ |
| 1/9 | 5 | 0 | 0 |
| Extruder pore size (μm) | | | |
| 0.1 | | 80 | 2+ |
| 1.0 | | 40 | 2+ |
| Not filtered | | 80 | 3+ |
| OMP source | | | |
| *N. meningitidis* 8047 (B:2b:P1.2) (WR) | 5 | 80 | 2+ |
| *N. meningitidis* 8765 (B:15:P1.3) (PM) | 5 | 80 | 2+ |
| *N. meningitidis* 8529 (B:15:P1.3) (CL) | 10 | 10 | 1+ |
| *N. gonorrhoeae* UU1X P1A (PM) | 10 | 80 | 2+ |

Table 3 Comparison of serological activity determined by ELISA and complement-dependent cytotoxicity (CDCX) in sera of mice immunized with GD3/proteosome vaccines

| GD3 antibody titre by ELISA | No. of mice | Median A at serum dilution 1/40 | Median CDCX titre: 20% Lysis endpoint (range) | Median CDCX titre: 50% Lysis endpoint |
|---|---|---|---|---|
| 1/320–1/640 | 7 | >1.80 | 1/80(1/20–1/320) | 1/5 |
| 1/80–1/160 | 8 | 0.42 | 1/40(0–1/80) | 0 |
| 1/40 | 4 | 0.16 | 1/5(0–1/20) | 0 |
| 0 | 5 | 0.08 | 0(0) | 0 |

*N. gonorrhoeae* UU1X P1A, appeared comparable but one batch prepared from Nm strain 8529 (B:15:P1.3) was significantly less effective.

### Specificity of GD3 antibodies induced by GD3/proteosome vaccines

The specificity of IgM anti-GD3 antibodies detected in the high titre sera of all mice was analysed by dot blot immune stains with ganglioside standards GM3, GM2, GM1, GD3, GD2, GD1a, GD1b and GT3. The results of one representative experiment are shown in *Figure 1*. Reactivity was generally restricted to GD3 ganglioside, though crossreactions with GT3 and GD3 amide as seen with anti-GD3 MmAb R24 were seen with occasional sera.

### Cell-surface reactivity and cytotoxicity of anti-GD3 antibodies

Anti-GD3 IgM antibodies induced by all vaccines were able to mediate complement-dependent lysis of tumour cells expressing GD3 in direct proportion to ELISA reactivity. This is shown for GD3/proteosome vaccine-induced antibodies in *Table 3*. GD3 negative cell lines were not lysed (data not shown). The titre of reactivity was slightly lower in cytotoxicity assays than titres detected by ELISA assays when the 20% lysis cutoff was used and significantly lower with the 50% lysis cutoff.

### Induction of antibodies to OMP

Unlike the antibodies induced against GD3 after immunization with GD3/proteosome vaccines, the antibody response against OMP was of higher titre, predominantly IgG and long-lasting, characteristic of T-cell dependent antigens. IgG antibodies against GD3 were not detected, IgM antibody titres against OMP were $< 1/80$. The OMP antibody response also differed from the GD3 antibody response in that the use of additional adjuvants magnified the antibody response against OMP but not GD3. In groups of ten mice each the median OMP ELISA titre rose from 1/1200 after vaccination with GD3 proteosomes alone, to 1/14000 with the use of SAF-m (containing MDP and L121), L121 alone or QS21. The GD3 antibody response remained exclusively IgM at a median titre of 1/160–1/320 in all groups. Comparison of the antibody responses seen against the GD3 and OMP components of the GD3/proteosome vaccine is shown in *Figure 2*.

### Delayed-type hypersensitivity (DTH) responses against GD3 and OMP

Mice were tested for DTH reactivity to GD3 and OMP by footpad injection 1–2 weeks after the third immunization. No significant DTH responses against GD3 were seen. DTH responses against OMP were seen in most mice immunized with proteosomes (median DTH response 0.6 mm). These reactions were significantly increased in mice receiving SAF-m, L121 or QS21 adjuvants (median DTH responses 1.0, 0.9 and 1.2 mm, respectively).

## DISCUSSION

Two of the ten immunological adjuvants tested, proteosomes and *S. minnesota* mutant R595, were particularly effective at augmenting antibody responses against the ganglioside GD3. We have already shown that R595 is not an effective vehicle/adjuvant for augmenting the immune response against gangliosides in patients[10] and so focus here on proteosomes. We demonstrate that the methods of OMP purification and proteosome formation, and a GD3/OMP ratio of approximately 2/1, are important for an optimal GD3/proteosome formulation. Although IgG antibody was not seen, consistent IgM antibody capable of mediating complement-dependent cytotoxicity was consistently induced. Repeated immunizations at short or long intervals and the use of additional adjuvants had no impact on the titre of IgM antibodies and failed to provoke an IgG antibody or DTH response against GD3. We have noted this T cell-independent pattern of antibody response previously against gangliosides in mice immunized with other ganglioside vaccines[9] and in patients immunized with GM2/BCG vaccines[8]. However, OMP are known to be B-cell mitogens in humans and to be well tolerated, so our data suggest that ganglioside/proteosome vaccines may be more immunogenic and less toxic than ganglioside/BCG vaccines.

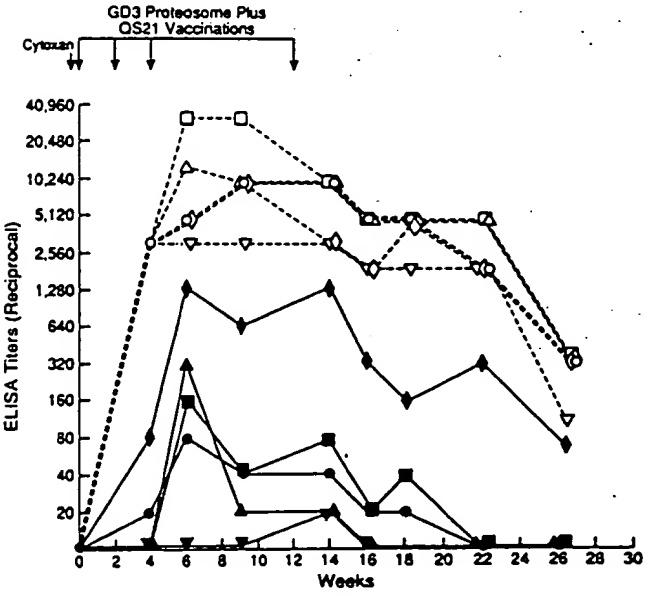


Figure 2 IgM responses against GD3 (closed symbols) and IgG antibody responses against OMP (open symbols) in the same mice immunized with GD3/proteosome vaccines. Each symbol shape represents an individual mouse. Arrows indicate time of cyclophosphamide and vaccine injections

The complete mechanism of action for proteosomes, as with most adjuvants, remains unknown. Clearly proteosomes provide a depot effect resulting in concentration and alignment of GD3 as well as prolongation of tissue half-life, thereby making GD3 more accessible to antigen processing and presenting cells. No direct role of OMP or proteosomes on macrophage activation has been described. The OMP in proteosomes are potent T cell-dependent antigens as seen by the high-titre IgG antibody and DTH responses induced against OMP. OMP are not known to be T-cell mitogens, but there is some evidence that OMP may be able to stimulate both second signal activity in B cells resulting in augmentation of T-cell activation (L. Wetzler, personal communication) and B-cell presentation to T-cell clones (G. H. Lowell, unpublished results). OMP have the additional advantage of being B-cell mitogens in both LPS-sensitive and -resistant mice[13] and, unlike LPS, they are mitogens and polyclonal activators of human B cells[13]. These properties may explain the high-titre IgM and IgG antibodies induced against peptides and meningococcal group B polysaccharide after immunization with the respective proteosome vaccines[11–14]. They were not, however, in themselves sufficient to induce IgG antibodies against GD3, probably because GD3 is an autoantigen in the mouse[5] to which the host is partially immunologically tolerant. We have shown previously that synthetic congeners of GD3, such as GD3 amide, which are not naturally expressed, are more effective immunogens, resulting in more consistent IgG antibody production in the mouse and in humans[24,25]. These IgG antibodies remain of relatively low titre despite repeated immunizations at various intervals, presumably because the immunogenic epitope of GD3 is a carbohydrate and carbohydrates generally induce only low-titre IgG antibodies. This may be because carbohydrates are not known to bind to major histocompatibility complex (MHC) for presentation to T cells or to induce T-cell immunity. The basis for the striking increase in anti-OMP antibody titres with the use of additional potent adjuvants such as pleuronic block copolymer L121, QS21 and SAF-m in the absence of any effect on GD3 antibody titres remains unclear. It is possible that these additional adjuvants augment helper T cells for OMP antibody induction but that this had no impact on GD3 which was not covalently attached to these same proteins and so would not be expected to remain in contact with them following antigen processing and presentation.

The effectiveness of these GD3/proteosome formulations in consistently inducing IgM antibodies in mice able to induce complement lysis of tumour cells expressing GD3, their lack of toxicity in these experiments, and the known safety of OMP vaccines in humans suggest that ganglioside/proteosome vaccines should now be tested in humans where they may be at least as immunogenic as ganglioside plus BCG vaccines and less toxic. They offer a promising alternative for augmenting the immunogenicity of cell-surface antigen-specific vaccines in cancer immunotherapy.

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## REFERENCES

1 Natoli, E.J. Jr, Livingston, P.O., Cordon-Cardo, C., Pukel, C.S., Lloyd, K.O., Wiegandt, H. *et al.* A murine monoclonal antibody detecting *N*-acetyl and *N*-glycolyl GM2: characterization of cell surface reactivity. *Cancer Res.* 1986, **46**, 4116–4120

2 Tsuchida, T., Saxton, R.E. and Irie, R.F. Gangliosides of human melanoma: GM2 and tumorigenicity. *J. Natl Cancer Inst.* 1987, **78**, 55–59

3 Dubois, C., Magnani, J.L., Grunwald, G.B., Spitalnik, S.L., Trisler, G.D., Nirenberg, M. and Ginsburg, V. Monoclonal antibody 1888, which detects synapse-associated antigens, binds to ganglioside GT3 (II³(NeuAc)₂LacCer). *J. Biol. Chem.* 1986, **261**, 3826–3830

4 Hamilton, W.B., Helling, F., Lloyd, K.O. and Livingston, P.O. Ganglioside expression on human malignant melanoma detected by immune thin layer chromatography. *Proc. Am. Assoc. Cancer Res.* 1992, **33**, 335

5 Livingston, P.O., Ritter, G. and Jones Calves, M. Antibody response after immunization with the ganglioside GM1, GM2, GM3, GD2 and GD3 in the mouse. *Cancer Immunol. Immunother.* 1989, **29**, 179–184

6 Houghton, A.N., Mintzer, D., Cordon-Cardo, C., Welt, S., Fleigel, B., Vadhan, S. *et al.* Mouse monoclonal IgG3 antibody detecting GD3 ganglioside: A phase I trial in patients with malignant melanoma. *Proc. Natl Acad. Sci. USA* 1985, **82**, 1242

7 Cheung, N.-K.V., Lazarus, H., Miraldi, F.D., Abramowsky, C.R., Kallick, S., Saarinen, U.M. *et al.* Ganglioside GD2 specific monoclonal antibody 3F8: a phase I study in patients with neuroblastoma and malignant melanoma. *J. Clin. Oncol.* 1987, **5**, 1430–1440

8 Livingston, P.O., Ritter, G., Srivastava, P., Padavan, M., Jones Calves, M., Oettgen, H.F. and Old, L.J. Characterization of IgG and IgM antibodies induced in melanoma patients by immunization with purified GM2 ganglioside. *Cancer Res.* 1989, **49**, 7045–7050

9 Livingston, P.O., Jones Calves, M. and Natoli, E.J. Jr. Approaches to augmenting the immunogenicity of the ganglioside GM2 in mice: purified GM2 is superior to whole cells. *J. Immunol.* 1987, **138**, 1524–1529

10 Livingston, P.O., Natoli, E.J. Jr, Jones Calves, M., Stockert, E., Oettgen, H.F. and Old, L.J. Vaccines containing purified GM2 ganglioside elicit GM2 antibodies in melanoma patients. *Proc. Natl Acad. Sci. USA* 1987, **84**, 2911–2915

11 Lowell, G.H., Smith, L.F., Seid, R.C. and Zollinger, W.D. Peptides bound to proteosomes via hydrophobic feet become highly immunogenic without adjuvants. *J. Exp. Med.* 1988, **167**, 658–663

12 Lowell, G.H., Ballou, W.R., Smith, L.F., Wirtz, R.A., Zollinger W.D. and Hockmeyer, W.T. Proteosome-lipopeptide vaccines: enhancement of immunogenicity for malaria CS peptides. *Science* 1988, **240**, 800–802

13 Lowell, G.H. Proteosomes, hydrophobic anchors, iscoms and liposomes for improved presentation of peptide and protein vaccines. In *New Generation Vaccines* (Eds Woodrow, G.C. and Levine, M.M.) Marcel Dekker, NY, 1990, p. 141

14 Zollinger, W.D. New and improved vaccines against meningococcal disease. In *New Generation Vaccines* (Eds Woodrow, G.C. and Levine, M.M.) Marcel Dekker, NY, 1990, p. 325

15 Canah, L.D., Irie, R.F., Singh, R., Cassidenti, A. and Paulson, J.C. Identification of a human neuroectodermal tumor antigen (OFA-I-2) as ganglioside GD2. *Proc. Natl Acad. Sci. USA* 1982, **79**, 7629

16 Yokochi, T., Nakashima, I., Kato, N., Miyadai, T., Yoshida, K. and Kimura, Y. Adjuvant actions of polyclonal lymphocyte activators. *Cell. Immunol.* 1985, **93**, 431–437

17 Byars, N.E. and Allison, A.C. Adjuvant formulation for use in vaccines to elicit both cell-mediated and humoral immunity. *Vaccine* 1987, **5**, 223

18 Kensil, C.R., Patel, U., Lennick, M. and Marciani, D. Separation and characterization of saponins with adjuvant activity from *Quillaja saponaria* Molina Cortex. *J. Immunol.* 1991, **146**, 431–437

19 Nakashima, I., Nagase, F., Yokochi, T., Ohta, M. and Kato, N. Adjuvant actions of polyclonal lymphocyte activators. *Cell. Immunol.* 1979, **46**, 69–76

20 Wetzler, L.M., Blake, M.S. and Gotschlich, E.C. Characterization and specificity of antibodies to protein I of *Neisseria gonorrhoeae* produced by injection with various protein I-adjuvant preparations. *J. Exp. Med.* 1988, **168**, 1883–1897

21 Lowry, O.H., Rosebrough, N.J., Farr, [redacted]nd Randall, R.J. Protein measurement with folin phenol reage[redacted]. *Biol. Chem.* 1951, **193**, 265–275

22 Svennerholm, L. Quantitative estimation of sialic acids – II. Colorimetric resorcinol-hydrochloric acid method. *Biochim. Biophys. Acta* 1942, **24**, 604–611

23 Ritter, G., Boosfeld, E., Markstein, E., Yu, R.K., Ren, S., Oettgen, H.F. *et al.* Biochemical and serologic characteristics of natural 9-*O*-acetyl GD3 from human melanoma, bovine buttermilk and chemically *O*-acetylated GD3. *Cancer Res.* 1990, **50**, 1403

24 Ritter, G., Boosfe[redacted], Calves, M.J., Oettgen, H.F., Old, L.J. and Livingston, P.O. [redacted]ubody response after immunization with gangliosides GD3, GD3 lactones, GD3 amide and GD3 gangliosidol in the mouse. GD3 lactone I induces antibodies reactive with human melanoma. *Immunobiology* 1990, **182**, 32–43

25 Ritter, G., Boosfeld, E., Adluri, S., Calves, M., Oettgen, H.F., Old, L.J. and Livingston, P.O. Antibody response to immunization with ganglioside GD3 and GD3 congeners (lactones, amide and gangliosidol) in patients with malignant melanoma. *Int. J. Cancer* 1991, **48**, 379–385